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Fine Structure of Spermatozoa of the Hagfish *Eptatretus burgeri* (Agnatha)[1]

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Abstract. Live motile spermatozoa of the hagfish *Eptatretus burgeri* were obtained for the first time, and their fine structure was examined. The spermatozoon is characterized by an extremely long midpiece. Two of the four midpiece mitochondria are extensively elongated and extend through almost the entire length of the tail. The acrosome contains electron-dense and less dense materials in two different compartments. Amorphous subacrosomal material lies between the acrosome and the nucleus. No distinct perforatorium rod or filamentous structure was observed within the subacrosomal material. Two centrioles lie almost end to end in the nuclear fossa near the posterior end of the nucleus. The structure of the acrosomal complex in the hagfish, which is quite different from that in the lamprey, was compared to that of other chordates with respect to its function in sperm-egg interaction and phylogeny.

Introduction

The reproductive life of the lamprey has been studied extensively (Kille, 1960; Nicander *et al.*, 1968), but little is known about reproduction, especially fertilization, in hagfish (Dean, 1899; Walvig, 1963; Gorbman, 1983). The structures of the testes and spermatozoa of hagfish have been described in *Myxine glutinosa, Bdellostoma burgeri,* and *B. stouti* (Walvig, 1963); *M. glutinosa* (Nicander, 1970); *E. burgeri* (Patzner, 1977, 1982); *Paramyxine atami* (Patzner, 1982); and *E. stouti* (Gorbman, 1990). Electron micrographical studies on the formation and structure of hagfish spermatozoa in *Myxine circifrons, M.* sp., *Eptatretus stoutii, E. deani,* and *E.* sp. were performed by Jespersen (1975). Because of difficulties in catching mature hagfish males alive (Walvig, 1963; Jespersen, 1975; Patzner, 1982), there had been few studies describing live and motile spermatozoa (Patzner, 1982).

In the sea near the Misaki Marine Biological Station in the Kanagawa Prefecture of Japan, the hagfish species *Eptatretus burgeri* migrates from depths of 50-100 m to the shore (10–12 m deep) between November and June (Kobayashi *et al.*, 1972); in contrast, most other hagfish species inhabit the deeper sea throughout the year (Adam and Strahan, 1963; Jespersen, 1975). *Eptatretus burgeri* is thought to breed in October, while living in deep water (Kobayashi *et al.*, 1972; Patzner, 1977, 1978).

By catching hagfish from the shore and keeping them in an aquarium (Fernholm, 1975; Ooka-Souda *et al.*, 1985), we were able to obtain males with mature spermatozoa and to investigate for the first time the ultrastructural details of these motile spermatozoa. Because the phylogeny, as well as the fertilization, of living agnathans (hagfish and lampreys) has attracted much attention from biologists (Brodal and Fänge, 1963; Hardisty, 1979), we compared these sperm to those of other chordates.

Materials and Methods

Males of the hagfish *Eptatretus burgeri,* 45–60 cm in body length, were caught in July in Sagami Bay near the Misaki Marine Biological Station in Kanagawa Prefecture, Japan. They were kept in a seawater tank (15°C) without food under controlled light (light 0700–1900) until they were used for experiments between September and December in the same year. After an animal was anesthesized with 0.5% 3-aminobenzoic acid ethyl ester (MS222, Sankyo Pharmaceut., Tokyo), its abdomen was opened to remove the testis. Actively motile spermatozoa were

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obtained when pieces of the posterior portion of the testis were immersed in seawater.

For transmission electron microscopy (TEM), small pieces of testis with mature spermatozoa were fixed for 1 h with 2% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4), and postfixed with 1% osmium tetroxide in the same buffer for 1 h. Dehydration in a graded alcohol series, followed by propylene oxide, and infiltration with Epon were performed using an automatic electron microscopy processor (REM-20B, Sakura, Tokyo). All procedures were done at room temperature. Thin sections were obtained using diamond knives, stained with uranyl acetate and lead citrate, and then examined with an electron microscope (JEOL100 or JEM-1200EX, JEOL Ltd., Tokyo).

For scanning electron microscopy (SEM), a testis was minced and spermatozoa were released onto a sheet of filter paper. Sperm on the paper were fixed in 2% glutaraldehyde in 70% seawater, followed by postfixation with 2% osmium tetroxide in 2.5% $NaHCO_3$. Samples were critical-point dried and examined using a scanning electron microscope (US4, JEOL Ltd., Tokyo).

Results

Macroscopic aspects of testis

Four male hagfish kept in the laboratory had mature testes between late September and early October in 1992. The testis appeared as a single nodular, spiral structure, longitudinally located along the right side of the mesentery as described in *E. burgeri* (Patzner, 1982) and in other hagfish (Walvig, 1963; Gorbman, 1990). Toward December, after the breeding season, the testis became thinner.

Motility and light microscopic aspects of sperm

When a small piece of mature testis was immersed in seawater, many spermatozoa were released from the testis and exhibited active forward motility for about 10 min. Motile spermatozoa could be obtained during late October in some fish. The number of spermatozoa with normal ultrastructure decreased in November. Thus, the hagfish kept in the laboratory had mature spermatozoa at about the same time as their natural breeding period.

The sperm head and tail could be identified with a light microscope. They were about 10 μm and 35–40 μm in length, respectively. These were almost the same as those observed by Walvig (1963). After active swimming in seawater for about 5 min, some spermatozoa stuck to the glass surface with the tip of their heads, rotating their tails freely. Some spermatozoa had shortened tails with a knot near the end of the tail. Heads of some spermatozoa were bent backward, and they swam with their heads pointing backward.

Fine structure of spermatozoa

Figure 1 is a scanning electron micrograph of an *E. burgeri* spermatozoon. The head is 8–10 μm in length and about 0.5 and 1.2 μm wide at the anterior and posterior regions, respectively. The acrosome is at the anterior end of the sperm head (Figs. 2 and 3a). The outer acrosomal membrane is in close approximation with the overlying plasma membrane at the posterior 1/2–2/3 of the acrosome (Fig. 3a). The inner acrosomal membrane covers a conspicuous subacrosomal material into which the apex of the nucleus projects, and the double structure of the nuclear membranes is indistinct in this area. The acrosomal contents are tightly packed in the anterior region of the acrosome (Fig. 3b), but somewhat loosely packed

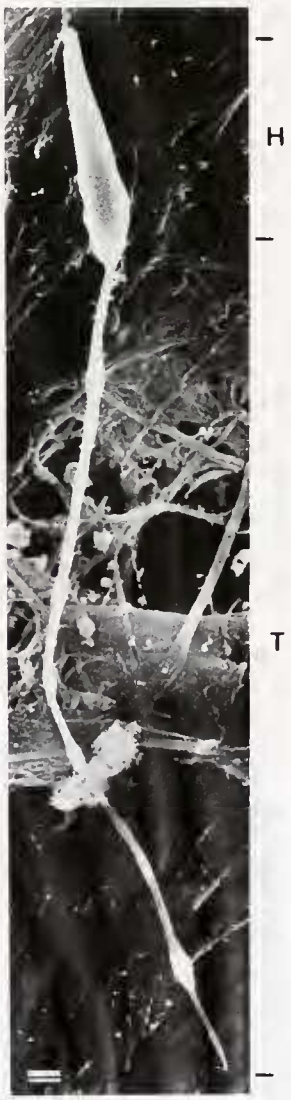

Figure 1. A scanning electron micrograph of an *Eptatretus burgeri* spermatozoon. H, head (acrosomal and nuclear regions); T, tail (midpiece and end piece). Bar = 1 μm.

Figure 2. An electron micrograph of the longitudinal section of the sperm head. A, acrosomal region; N, nucleus. Bar = 1 μm.

in the posterior region (Fig. 3c,d). The subacrosomal material between the acrosome and the nucleus is almost homogeneous in electron density. Neither a distinct perforatorium rod nor filamentous structures are detected within the subacrosomal material.

The nucleus increases its thickness posteriorly (Fig. 2). In the posterior lateral surface of the sperm head is a small fossa in which two centrioles are located almost end to end (Fig. 4a). The axoneme has the ordinary 9 + 2 arrangement of doublets (Fig. 4e,f).

Four mitochondria encircle the axoneme at the base of the flagellum (Fig. 4c), each arranged longitudinally (Fig. 4a). Two of them are extensively elongated and extend almost along the entire length of the axoneme (Fig. 4d,e). Most cross sections of the midpiece exhibit a 9 + 2 arrangement of axonemal doublets flanked by two mitochondria (Fig. 4e). Axonemes without associated mitochondria or with only one mitochondrion are observed only in sections near the posterior end of the tail (Fig. 4f).

The unique feature of the *E. burgeri* spermatozoon is the two extremely elongated mitochondria that run parallel with the axoneme throughout nearly the entire length of the tail. Figure 5 diagrams the structure of various regions of the *E. burgeri* spermatozoon.

Discussion

Spermatozoa of species that exhibit external fertilization, including common fishes, usually have a few mitochondria in the short midpiece surrounding the centrioles and the 9 + 2 axoneme without accessory structures in the flagellum. The sperm midpiece in many species with internal fertilization [*e.g.*, mammals (Phillips, 1977) and viviparous teleosts (Grier, 1975)] has a long mitochondrial sheath or a long cytoplasmic sleeve that contains many separate mitochondria. Hagfish sperm have four mitochondria, but two of them extend nearly the entire length of the tail, forming a long midpiece, as in spermatozoa of species with internal fertilization.

The acrosomal vesicle of protochordate spermatozoa is either ovoid, as in the urochordate *Oikopleura* (Holland *et al.*, 1988), or cap-shaped, as in the cephalochordate *Branchiostoma* (Baccetti *et al.*, 1972), and a distinct ac-

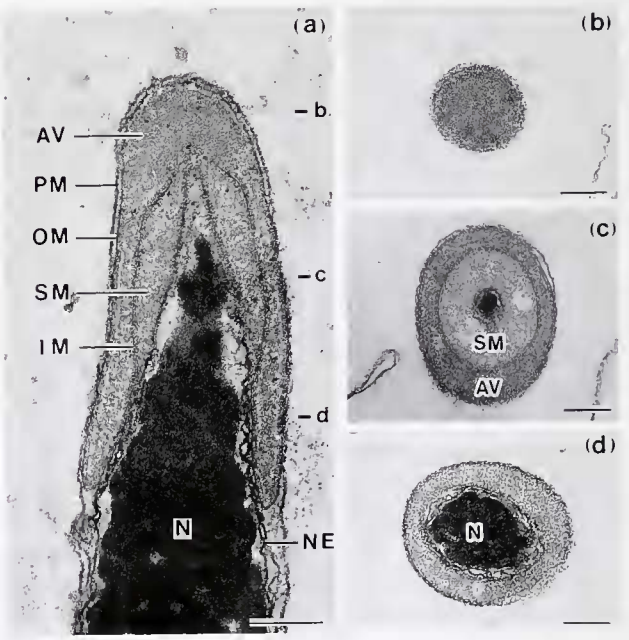

Figure 3. Longitudinal (a) and cross sections (b–d) of the acrosomal region. Labels -b, -c, and -d in figure (a) indicate the levels of sections shown in b, c, and d, respectively. Acrosomal vesicle (AV) and the underlying subacrosomal material (SM) cover the anterior end of the nucleus (N). IM, inner acrosomal membrane; NE, nuclear envelope; OM, outer acrosomal membrane; PM, plasma membrane. Each bar = 200 nm.

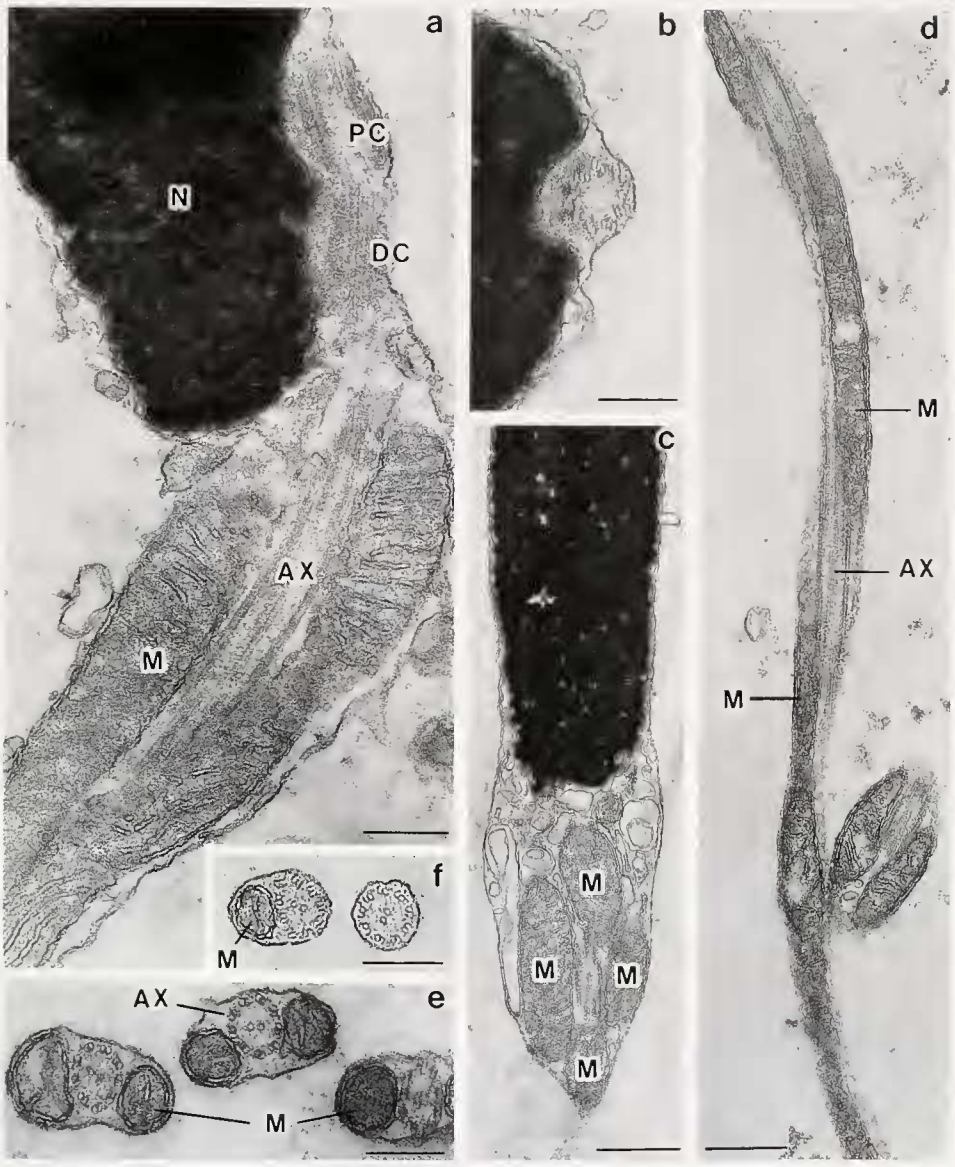

Figure 4. Longitudinal and cross sections of various regions of the tail. (a) The base of a flagellum. The proximal centriole (PC) and distal centriole (DC) lie almost longitudinally. Elongated mitochondria (M) are arranged along the axoneme (AX). (b) A cross section through a centriole. (c) An oblique section through the base of a flagellum. Four mitochondria encircle the axoneme. (d) A longitudinal section of the winding and twisting flagellum. Two long mitochondria flank the axoneme. (e) Cross sections of three flagellae. Two mitochondria flank the axoneme in the plane of two central singlets. (f) Cross sections of flagellae through near endpiece (left) and endpiece (right). Note that the axoneme without mitochondria has incomplete doublets. Bar = 250 nm (in a, b, e, f) and 500 nm (in c, d).

rosomal process is produced *de novo* following the acrosomal exocytosis at the anterior end of the sperm (Holland *et al.*, 1988). Spermatozoa of vertebrates such as amphibians (Yoshizaki and Katagiri, 1982; Fig. 6c), reptiles (Furieri, 1970; Fig. 6d), birds (Okamura and Nishiyama, 1978; Fig. 6e), and mammals (Yanagimachi and Noda, 1970; Fig. 6f) have a cap-shaped acrosomal vesicle and underlying subacrosomal material, which cover the anterior portion of the nucleus. The exocytosis of the acrosomal vesicle occurs at several points, and a new acrosomal process does not protrude (Yanagimachi and Usui, 1974; Okamura and Nishiyama, 1978; Yoshizaki and Katagiri, 1982).

A variety of acrosomal structures are found in fish spermatozoa. The lamprey, which is a cyclostome, has spermatozoa that carry a spherical acrosomal vesicle at

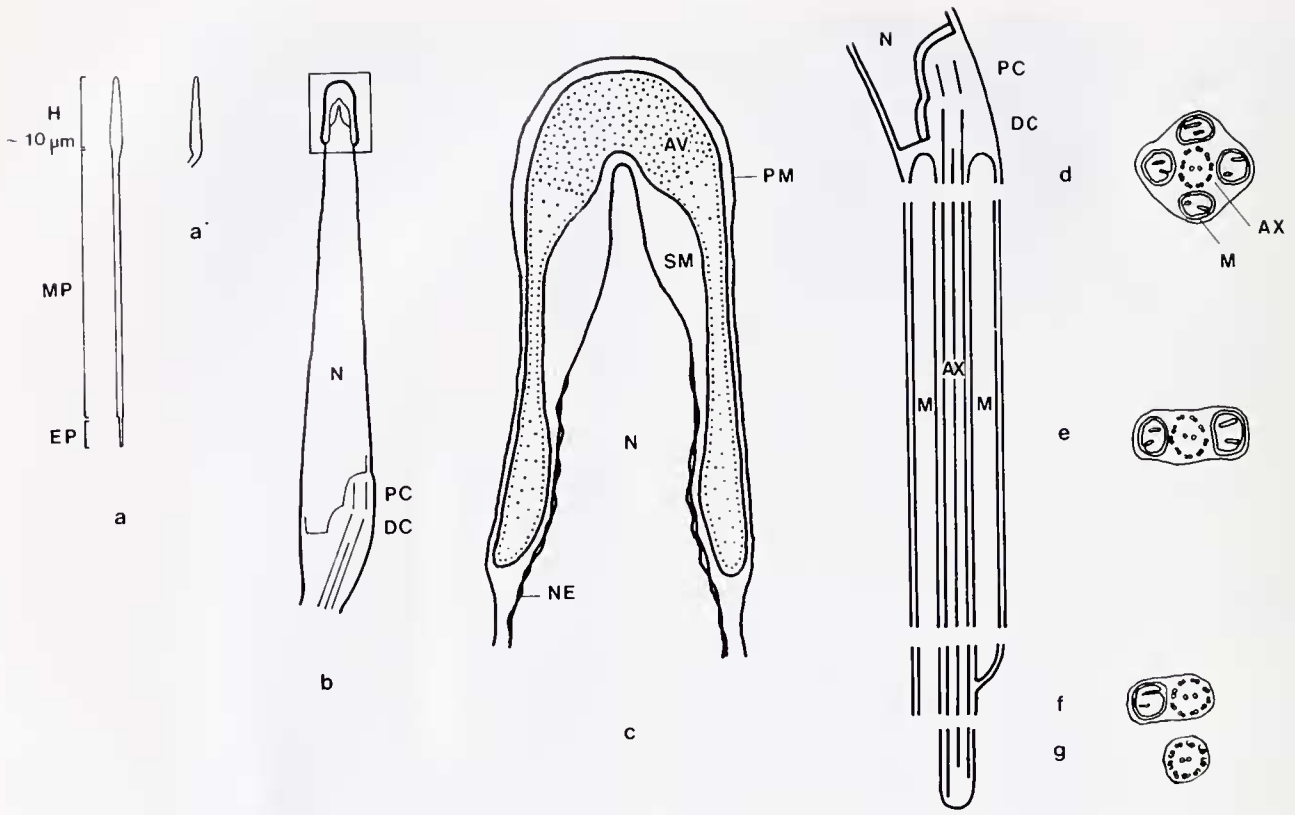

Figure 5. Schematic drawings of the *Eptatretus burgeri* spermatozoon. (a) Whole view at low magnification; a, surface view; a', side view of the head. (b) Head and regions. (c) Acrosomal region indicated by a rectangle in the figure b. (d–g) A longitudinal and cross section of the flagellum. AV, acrosomal vesicle; AX, axoneme; DC, distal centriole; EP, endpiece; H, head; M, mitochondria; MP, midpiece; N, nucleus; NE, nuclear envelope; PC, proximal centriole; PM, plasma membrane; SM, subacrosomal material.

the anterior end of their heads, subacrosomal material between the acrosome and the nucleus, and a long perforatorium rod through the nucleus (Follenius, 1965; Stanley, 1967; Nicander and Sjöden, 1971; Jaana and Yamamoto, 1981) (see Fig. 6b). When the sperm reach the outer chorion of the egg during fertilization (Nicander and Sjöden, 1971), or when they are exposed to fixatives (Jaana and Yamamoto, 1981), a long acrosomal process is formed. Spermatozoa of the elasmobranch *Squalus suckleyi*, a species that has internal fertilization, have a cap-shaped acrosomal vesicle and a subacrosomal rod (Stanley, 1971). Spermatozoa of the sturgeon, *Acipenser transmontanus*, which have a scalloped and cap-shaped acrosomal vesicle and filamentous structure in the subacrosomal material and in the canals through the nucleus, form an acrosomal process upon the acrosome reaction (Cherr and Clark, 1984). In Holostei (Afzelius, 1978) and Teleostei (Mattei, 1970), spermatozoa lack an acrosome. In hagfish, the acrosomal vesicle of the spermatozoa covers the protrusion of the nucleus with underlying subacrosomal material (Figs. 3, 6). In our preliminary experiments, the acrosomal exocytosis of the *Eptatretus burgeri* sperm occurred not only at the apical point of the sperm head but at several points, and was not followed by conspicuous formation of a long process. Such features are common in the spermatozoa of higher animals. The role of the acrosomal complex of the hagfish spermatozoa remains to be studied.

The acrosome reaction occurs inside or on the surface of the egg envelope to allow sperm penetration. In the case of external fertilization in teleosts, spermatozoa reach the egg plasma membrane through a narrow micropyle that has been perforated in the chorion; the sperm lack an acrosome. Lamprey eggs have a two-layered chorion (Afzelius *et al.*, 1968) that has no micropyle (Kille, 1960); the sperm penetrate the chorion with the acrosome reaction (Nicander and Sjöden, 1971). Sturgeon eggs have numerous micropyles, and the sperm form an acrosomal process (Cherr and Clark, 1984). In hagfish eggs, one micropyle with an outer opening diameter of 4.2 μm, 4.7 μm, or 4 μm in *Myxine glutinosa* (Kosmath *et al.*, 1981), *Eptatretus burgeri* (Kosmath *et al.*, 1981), or *E. stouti* (Koch *et al.*, 1993), respectively, is perforated at the animal pole through the thick and hard chorion. Inasmuch as

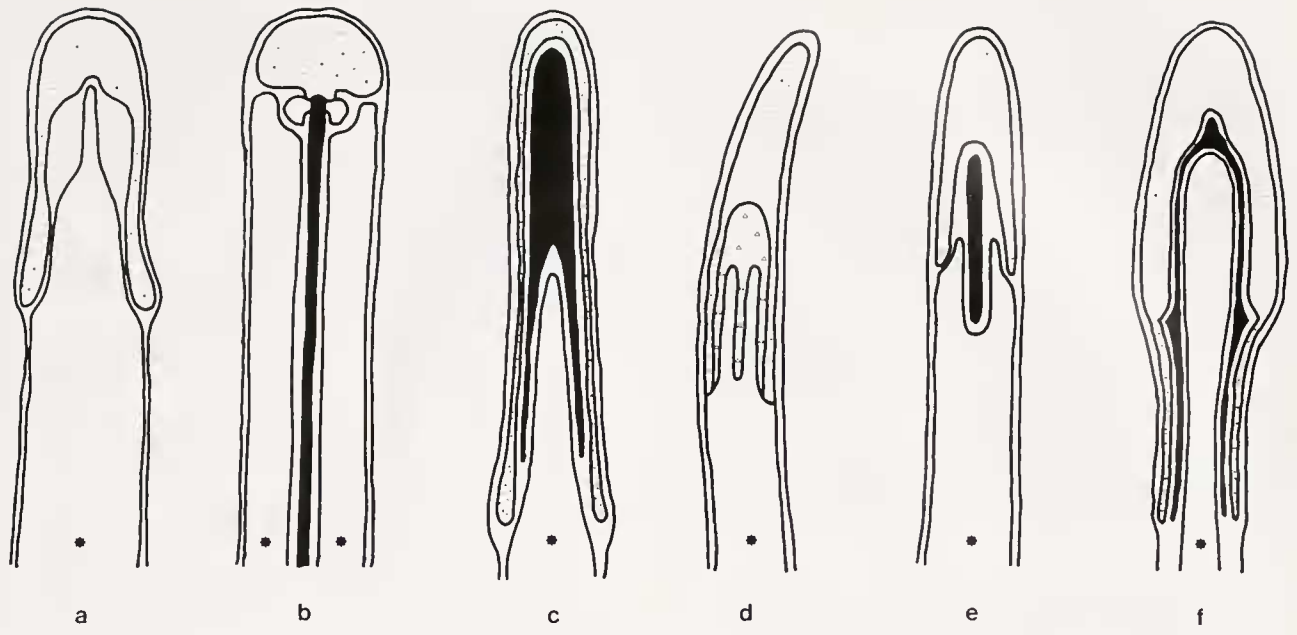

Figure 6. Comparison of the acrosomal region of the spermatozoa of various vertebrates. (a) Agnathans (*Eptatretus burgeri*); (b) agnathans (*Lampetra planeri*); (c) amphibians (*Bufo bufo japonicus*); (d) reptiles (*Chelonia* sp.); (e) birds (*Gallus gallus*); (f) mammals (*Mesocricetus auratus*). b–f were redrawn from Stanley (1967), Yoshizaki and Katagiri (1982), Furieri (1970), Okamura and Nishiyama (1978), Yanagimachi and Noda (1970), respectively. Acrosomal vesicle, spotted; perforatorium (subzonal material), shaded; nucleus, *; inner acrosomal cap with granular substance (Furieri, 1970), triangles.

the head of the hagfish spermatozoon is 2.5–3 μm wide in *M. glutinosa* (Walvig, 1963), about 1.2 μm wide (see Results) and 1.5 μm wide (Walvig, 1963) in *E. burgeri*, and about 1.5 μm wide (Jespersen, 1975) and 2.5–3 μm wide (Koch *et al.*, 1993) in *E. stouti*, the spermatozoa could access the egg surface directly. Judging by the features of the anterior portion of the spermatozoa and the micropyle, the relationship between sperm and egg in hagfish may differ from those in lampreys and teleosts. The exocytosis of the acrosomal vesicle at several points in hagfish (our preliminary observation) as seen in higher vertebrates may be different from the acrosome reaction in sturgeon. The relationship between the existence of egg micropyle and no acrosome in spermatozoa has been considered in teleosts having external fertilization (Baccetti and Afzelius, 1976). In hagfish and sturgeon, however, gametes have both structures, although the mode of fertilization in hagfish is still unknown. The structure of the micropyle in hagfish and sturgeon should be studied in detail to understand the role of these structures in fertilization.

The hagfish has a phylogenetically interesting position in the Chordata. Analysis of sperm function—for example, the interaction between sperm and egg during fertilization and the acrosome reaction—would contribute to an understanding of both the mode and the phylogenical aspects of fertilization in hagfish.

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